

UNITED STATES PATENT APPLICATION
FOR
BACTERIAL EFFECTS ON METAL ACCUMULATION BY PLANTS

DESCRIPTION OF THE INVENTION

Field of the Invention

[001] The present invention relates to enhancing the ability of certain plants to extract metals from soil, particularly by adding bacteria to the soil.

Background of the Invention

[002] Incorporated herein by reference to generally explain the nature of the art are the following: (1) Barber DA, Lee RB (1974), The effect of microorganisms on the absorption of manganese by plants. *New Phytology* 73: 97-106; (2) Chaney RL (1983), Plant uptake of inorganic waste constituents. In: Parr JF, Marsh PB, Kla JM, eds. *Land treatment of hazardous wastes*. Park Ridge, NJ, USA: Noyes Data Corporation, 50-76; (3) Salt DE, Krämer U (2000), Mechanisms of metal hyperaccumulation in plants. In: Raskin I, Ensley BD, eds. *Phytoremediation of toxic metals: using plants to clean up environment*. Sommerset, NJ, USA: John Wiley and Sons, Ltd., 231-246; (4) Kelley BC, Tuovinen (1988), Microbial oxidation of minerals in mine tailings. In: Solomons W, Foerstner V, eds, *Chemistry and biology of solid waste*. Berlin, Germany: Springer Verlag, 33-53; (5) Lynch JM, (1990), *The rhizosphere*. New York, USA: John Wiley and Sons; (6) Martin JP, Farmer WJ, Ervin JO (1973), Influence of steam treatment and fumigation of soil on growth and elemental composition of avocado seedlings. *Soil Science Society of America* 37: 56-60; (7) Crowley DE, Wang YC, Reid CPP, Szaniszlo PJ (1991), Mechanisms of iron acquisition from siderophores by microorganisms and plants. *Plant and Soil* 130: 179-198; (8) Smith SE, Read DJ (1997), *Mycorrhizal symbiosis*. San Diego, CA, USA: Academic Press; (9) Loper EJ, Henkels, MD (1999), Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Applied Environmental Microbiology* 65:

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bacteria that decrease heavy metal toxicity in plants, *Canadian Journal of Microbiology* 46: 237-245; (22) Phytoextraction of soil nickel using Alyssum species, In: Parr JF, Marsh PB, Kla JM, eds. *Land treatment of hazardous wastes*, Park Ridge, NJ, USA, Noyes Data Corporation, 50-76; (22) Phytoextraction of soil nickel using Alyssum species, In: Wentzel WW, Adriano DC, Alloway B, Doner HE, Keller C, Lepp NW, Mench M. Naidu R, Pierzynski GM, eds. *Proceedings of the Extended Abstracts from the 5th International Conference on the Biochemistry of Trace Elements (July 11-15, (1999), Vienna, Austria)*, Vienna, Italy: International Society of Trace Element Research, 14-15; (23) Ferriss RS, 1984, Effects of microwave oven treatment on microorganisms in soil, *Phytopathology* 74: 121-126; (24) Fletcher CL, Kaufman DD, (1980), Effects of sterilization methods on 3-chloronailine behavior in soil, *Journal of Agriculture Food Chemistry* 28: 667-671; (25) (Zn, Cd, Ni en al) en siderofoot-afhankelijke ijzer-opname in verschillende fluorescerende Pseudomonas stammen, Brussels, Belgium: Licentiaatsthesis, Departement Algemene Biologie; (26) Hassan MET, (1996), *Genetic mechanism of heavy metal resistance of Pseudomonas aeruginosa CMG103*, PhD thesis, University of Karachi, Pakistan; (27) Hu X, Boyer GL (1996), Siderophore-mediated aluminum uptake by *Bacillus megaterium* ATCC 19213, *Applied Environmental Microbiology* 62: 4044-4048; (28) Huyer M, Page W (1988), Zn²⁺ increases siderophore production in *Azotobacter vinelandii*, *Applied Environmental Microbiology* 54: 2615-2631; (29) Kukier U, Chaney RL (2001), Amelioration of Ni phytotoxicity in muck and mineral soils, *Journal of Environmental Quality* 30: 1949-1960; (30) Lasat MM (2002), Phytoextraction of toxic metals, a review of biological mechanisms, *Journal of Environmental Quality* 31: 109-120; (31) Lopes AS, Wollum AG (1976) Comparative effects of methyl bromide, propylene oxide, and autoclave sterilization on specific

soil chemical characteristics, *Turialba* 26: 351-355; (32) Neilands JB (1981), Microbial iron compounds, *Annual Review of Biochemistry* 50: 715-731; (33) Official Anal. Chem. (AOAC) Method (1984), 3 014(a), 14th edn. Gaithersberg, MD, USA: AOAC International, 38-40; (34) Peterson GH (1962), Microbial activity in heat and electron-sterilized soil seeded with microorganisms, *Canadian Journal of Microbiology* 8: 519-524; (35) Reasoner DJ, Geldreich EE (1985), A new medium for the enumeration and subculture of bacteria from potable water, *Applied Environmental Microbiology* 49: 1-7; (36) Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, Raskin I (1995), Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants, *Bio/Technology* 13: 468-474; (37) Skipper HD, Westermann DT (1973), Comparative effects of propylene oxide, sodium azide, and autoclaving on selected soil properties, *Soil Biological Biochemistry* 5: 409-414; (38) Whiting SN, De-Souza MP, Terry N (2001), Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*, *Environmental Science Technology* 35: 3144-3150; (39) Wolf DC, Dao TH, Scott HD, Lavy TL (1989), Influence of sterilization methods on selected soil microbiological, physical, and chemical properties, *Journal of Environmental Quality* 18: 39-44; and (40) Zhao FJ, Hamon RE, McLaughlin MJ (2001), Root exudates of the hyperaccumulator *Thlaspi caerulescens* do not enhance metal mobilization, *New Phytologist* 151: 613-620.

[003] The following U.S. patents are also incorporated herein by reference to show the state of the art: U.S. Patent No. 5,944,872 to Chaney, et al. entitled Method for Pytomining Nickel, Cobalt and Other Metals from Soil and U.S. Patent No. 5,711,784 to Chamy, et al. also entitled Method for Pytomining of Nickel, Cobalt and other Metals from Soil.

[004] A number of plant species are capable of accumulating concentrations of metals from soil, and such known plant species are endemic to metalliferous soils. Among the metals that can be accumulated in plants are nickel, zinc and cobalt and certain plants can accumulate these metals in their above ground biomass at levels greatly exceeding those normally considered to be phytotoxic. Such plants are termed metal “hyperaccumulators.” In the case of nickel (chemical symbol, “Ni”), hyperaccumulators are characterized by being able to accumulate concentrations greater than 1000 mg of Ni kg⁻¹ of shoot dried weight when such plants are grown in their natural habitat. More than 400 plant species have been identified as hyperaccumulators, of which 75% are Ni hyperaccumulators that grow on ultramafic soils.

[005] As a result of their ability to remove considerable quantities of metals from soils, hyperaccumulators are attracting interest from scientists interested in phytoremediation (also more generally called phytoextraction), which could be a cost-effective technique for decontamination of environments polluted by metals. Phytoremediation involves planting hyperaccumulators in soil contaminated with metal, or growing hyperaccumulators from seeds in the contaminated soil, and allowing the plants to extract the metal. The Ni-hyperaccumulating plant *Alyssum murale* has received the most attention because of its extraordinary ability to extract and accumulate Ni from soils.

[006] Hyperaccumulation of metals by some plants has been extensively investigated. Most of the research on hyperaccumulation has focused on the physiological mechanisms of transport of the plants, and the plants’ storage and tolerance of the accumulated metals. Considerably less information is available on the processes in the rhizosphere of hyperaccumulators. Root proliferation and

effective root uptake mechanisms are among the key processes in the rhizosphere that distinguish metal hyperaccumulators from normal plants. The roles of root exudates and microbial activity in the soil are largely unknown, although Zhao *et al.* (2001) has recently suggested that root exudates of the genus of plants from *Thlaspi* do not enhance mobilization of metals in soil.

[007] Microorganisms (also called microbes) are usually present in soils to which hyperaccumulators are native, even in those soils containing high concentrations of metals. Microorganisms (such as bacteria) in the soil may affect trace metal mobility and availability to the hyperaccumulator plant; they may produce iron chelators and siderophores for ensuring iron availability, reduce soil pH, and/or solubilize metal-phosphates. For example, chemolithotrophic bacteria have been shown to enhance environmental mobility of metal contaminants via soil acidification, or in contrast, to decrease their solubility due to precipitation as sulfides. Some microbes influence root parameters, such as root morphology and growth. An increase in root exudation of organic solutes could affect the rate of phytosiderophore release. In turn, rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake.

[008] Any practice that increases metal (such as Ni) uptake into plants during phytomining would increase the commercial value of using metal-extracting plants, particularly hyperaccumulators, and thus the ultimate success of phytomining and phytoremediation.

SUMMARY OF THE INVENTION

[009] The present invention relates to methods for enhancing the ability of plants to extract metals from soil. As used herein, the term “metal-extracting plant” means a plant that can extract metal from soil, and includes hyperaccumulators. As

used herein, the term “enhancing-bacteria” means any microbe that enhances the ability of a metal-extracting plant to extract metal from soil and includes rhizobacteria. Such enhancement means that the metal-enhancing plant extracts more metal from the soil utilizing a method according the invention than it would if the invention were not utilized.

[010] A method according to the invention is preparing soil including metal to be extracted by adding to the soil enhancing-bacteria that enhances the ability of a metal-extracting plant to extract the metal from the soil.

[011] Another method according to the invention is extracting metal from soil, wherein the method comprises the steps of adding an enhancing-bacteria to the soil, and planting one or more metal-extracting plants to the soil to extract the metal, wherein the ability of the metal-extracting plants to extract the metal is enhanced by the presence of the enhancing-bacteria in the soil. Preferably the metal-extracting plants are added to the soil as seeds and preferably the seeds have been inoculated with the enhancing-bacteria.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

[012] The following detailed description is for the purpose of setting forth exemplary embodiments of the invention, and is not meant to limit the scope of the invention. As previously mentioned, some plants, such as *Alyssum murale* (“*A. Murale*”), have a remarkable ability to hyperaccumulate metals, such as Ni, from soils containing mostly nonlabile metals (particularly Ni). The present inventors have found that microbes, particularly certain rhizobacteria, enhance metal accumulation (and hence, extraction) by plants, for example Ni extraction by *A. murale*, and this phenomenon is believed by the inventors to be caused by the

ability of the microbes to increase the availability to the plant of metal in soil, although the invention is not limited to any particular theory.

[013] In the example herein, three bacteria, *Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, and *Microbacterium arabinogalactanolyticum* were added to both sterile and nonsterile Ni-rich, serpentine soil collected from OR, USA. *Sphingomonas macrogoltabidus* reduced Ni extraction by 10 mM $\text{Sr}(\text{NO}_3)_2$ from soil. *M. arabinogalactanolyticum* significantly increased Ni extraction, whereas *M. liquefaciens* had no effect except for when plant seeds were inoculated with *M. liquefaciens*. Extractability of few other metals was affected by inoculation of the soil with the enhancing-bacteria (i.e., adding the bacteria to the soil). When these three bacteria were also added to surface-sterilized seeds of *A. murale* grown in nonsterile soil, they increased Ni uptake into the shoot by, respectively, 17% (*S. macrogoltabidus*), 24% (*M. liquefaciens*), and 32.4% (*M. arabinogalactanolyticum*), compared with uninoculated soil controls.

[014] Example 1

[015] Soil sampling and preparation:

[016] A nickel-rich, serpentine soil was collected near Cave Junction, OR, USA. Samples of this soil were mixed in a large container and dried at room temperature, then were crushed, sieved, and analyzed by the soil testing laboratory of the University of Maryland. Total metals and other physical and chemical properties of the soil are presented in Table 1. One half of the soil was sterilized (at 121°C) by autoclaving on three separate occasions for 1 hour each time. Table 1 shows the physical and chemical characteristics of this soil:

Table I

Sand	Silt	Clay	OM*	pH	Mg	P	K	Mn	Ca	Zn	Cu	Ni	Cu	CEC***
g kg ⁻¹					mg kg ⁻¹									meq100 g ⁻¹
48	24	28	4.1	6.4	172	32	48	37.5	178	14	22	4390	0.8	6.7

* Organic matter. ** Cation exchange capacity

[017] Extractable metals were measured by shaking 10 gram, air-dried sterile and nonsterile soil for 2 hours in 20 milliliters of 10 mM Sr(NO₃)₂, according to the teachings of Kukier U, Chancy RL. 2001. Amelioration of Ni phytotoxicity in muck and mineral soils, *Journal of Environmental Quality* 30: 1949-1960, the disclosure of which is incorporated herein by reference. Samples were filtered and acidified with HNO₃ before analysis. Metal concentrations were determined using both flame atomic absorption spectrometry (AAS), and inductively coupled plasma atomic emission spectrometry (ICP-AES) using yttrium as an internal standard.

[018] Bacterial solubilization of metals in soil:

[019] Selecting an enhancing bacterium for use in treating the soil from which metal will be removed is performed by a process involving the step of selecting the microbe by determining whether it will grow in an environment including relatively high concentrations of the metal to be removed from the soil. Other traits that may be used to select the enhancing-bacteria are its ability to: (1) thrive in phosphorus, (2) tolerate low soil pH, and/or (3) produce chelating agents.

[020] Three rhizobacterial strains were selected for study of their effect on Ni extraction from soil and effect on Ni uptake into *A. murale*. These bacteria were originally isolated from the rhizosphere of *A. murale* grown on the same Ni rich, serpentine soil from Oregon, USA. The methods of isolating such bacteria are known to those skilled in the art. A deposit of *M. arabingalatanolytcum* was made

with the American Type Culture Collection (ATCC) on August 21, 2002 on behalf of the University of Maryland and has Patent Deposit Designation PTA-4604.

[021] The specific characteristics of the three bacteria selected are as follows: *Sphingomonas macroglabridus* is a nonacid producer, non-phosphate solubilizer, nonsiderophore producer, and grows on 8 mM NiCl₂. *Microbacterium liquefaciens* is an acid producer, phosphate solubilizer, siderophore producer, and grows on 8 mM NiCl₂. *Microbacterium arabinogalactanolyticum* is an acid producer, phosphate solubilizer, nonsiderophore producer, and grows on 8 mM NiCl₂.

[022] Bacterial cells were grown overnight in 250-ml Erlenmeyer flasks containing 100 ml of sterilized R2A broth (Reasoner & Geldreich, 1985) on a shaker at 150 r.p.m. at 30°C until late log phase. Living and dead bacterial cells were then harvested by centrifugation (12 000 x g, 20°C, 10 min), and the pellets were washed twice with sterile distilled water. Bacterial suspensions in distilled water were adjusted to an absorbance at 600 nm of 0.5 (equivalent to approximately 7.4×10^8 CFU ml⁻¹). One ml of each bacterial suspension was used as an inoculum for 10 grams of autoclaved sterile and 10 grams of nonsterile soils in 50 ml Falcon tubes. Soils were incubated at room temperature for 18 days.

[023] Pot experiment:

[024] First, seeds of *Alyssum murale* were inoculated with a bacteria (i.e., bacteria was added to the seeds). The bacterial cells were grown in 500-ml Erlenmeyer flasks containing 250 ml of sterilized R2A (Reasoner & Geldreich, 1985) broth overnight, until late log phase. Dead cells were obtained by autoclaving at 121 C one flask of each bacterium for 20 minutes. Living and dead bacterial cells were collected as previously described, washed twice with sterile distilled water and

then suspended in sterile distilled water. Cell densities were adjusted to an absorbance at 600 nm of 0.5 and were used for seed inoculation.

[025] Before seeding, *A. murale* seeds were surface-sterilized by shaking in 70% ethanol for 5 min, followed by shaking in 2% sodium hypochlorite for 30 min. Sterile seeds were next washed in five, 5-mm rinses with sterile water.

[026] Surface sterilized *A. murale* seeds were sown in plastic pots containing 500 g Ni rich serpentine soil mixed with the same volume of perlite. Perlite was added to improve water and gas movement. Five milliliter (ml) of the appropriate bacterial suspension was added at a concentration of 10^8 Colony Forming Units (CFU) per ml of sterile distilled water, whereas for the noninoculated control plants, just the same amount of sterile distilled water was added. After 2 weeks, the plants were thinned to five plants per pot. Plants were grown for 2 months in a glasshouse at 25°C and a 16/8 day/night regime, meaning 16 hours of daylight followed by 8 hours of darkness. Tap water and nutrient solutions of KNO_3 $(\text{NH}_4)_2\text{SO}_4$ and $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ were added as needed. All treatments were replicated five times.

[027] Plant harvest and preparation:

[028] After two months, whole *A. murale* plants were removed from the pots. Shoot and roots were separated. Plant shoots were washed with deionized water, rinsed and dried at 65°C. Dry plant samples were weighed and ground using a Wiley Mill. Two grams or less were ashed at 480°C for 16 hours. The ash was digested with concentrated HNO_3 , dissolved in 3 molar (M) HCl, filtered and diluted to 25 ml with 0.1 M HCl (AOAC Official Method of Analysis 3.014 (a), 1984). Metal concentrations were determined using both AAS and inductively coupled plasma atomic emission spectroscopy (ICP-AES) using yttrium as an internal standard.

[029] Statistical analyses:

[030] Data were analyzed using the Statistical Analysis System ("SAS") version 6.12 (SAS Institute, Cary N.C). Treatment means (i.e., the average of replicates for each bacteria addition) were separated using the Waller-Duncan K-ratio f-test after it was determined that there was a significant (probability or $P < 0.05$) treatment effect using the GLM procedure, which is a statistical method that determines if treatment means are different from one another.

[031] Results:

[032] Peterson (1962) reported that colonization of autoclaved soil by soil microorganisms is inhibited and the duration of inhibition is related to the time of autoclaving and the type of microbe tested. Autoclaving soil destroys the free radical mechanisms involved in biotic transformation of certain organic compounds (Fletcher & Kaufman, 1980). Autoclaving soil has also been shown to influence soil chemistry. Particularly noteworthy is the large increase in extractable Mn, often to toxic levels, that can result from autoclaving soil (Martin *et al.*, 1973; Skipper & Westermann, 1973; Lopes & Wollum, 1976; Wolf *et al.*, 1989). Autoclaving soil has also been reported to influence extractable Al and Fe and to decrease trace element levels but generally does not result in significant changes in Ca, Mg, or K levels (Ferriss, 1984).

[033] Soil sterilization significantly reduced $\text{Sr}(\text{NO}_3)_2$ extractable Ni compared with nonsterilized soils, as shown in Table 2. On the other hand, autoclaving increased extractable Fe, Mn, and Co. Autoclaving had no significant effect on other elements examined. Table 2 shows the effect of autoclaving on extractable metal content using strontium nitrate.

Table 2

Soil	Ni	Zn	Cu	Ca	Fe	Mg	Mn	Co	K	P
	mg kg ⁻¹	dry soil								
Nonsterile	2.17 ^a	0.48 ^a	< DL	177.4 ^a	0.01 ^b	114.2 ^a	3.6 ^b	0.00 ^b	21.45 ^a	0.86 ^a
Sterile	1.55 ^b	0.47 ^a	< DL	152.6 ^b	0.07 ^a	103.4 ^a	40.7 ^a	0.24 ^a	20.15 ^a	0.99 ^a

< DL = below detection limit. *Means followed by different letters within the same column are significantly different at $P < 0.05$.

[034] Thus, in the current study, autoclaving the soil increased extractable soil Mn from 3.6 to 40.7 mg kg⁻¹.

[035] To study the influence of the three bacterial isolates on metal solubilization in the serpentine soil, the bacteria were inoculated into sterile and nonsterile soil and extractable metal concentrations were determined at 6 weeks post inoculation. In sterilized soil there were few significant effects of bacterial inoculation on Sr(NO₃)₂ extractable concentrations of any of the elements examined, as shown in Table 3. *Microbacterium liquefaciens* significantly increased Fe solubilization and decreased the concentration of extractable Mn in sterile soils compared with the sterile uninoculated soils, as shown in Table 3. *Sphingomonas macrogoltabidus* decreased extractable Ca, Mg, and K. *Microbacterium arabinogalactanolyticum* had no effect on extraction of any of the elements examined. Table 3 shows the effect of different bacterial isolates on metal solubilization in sterilized Ni rich soils.

Table 3

Isolate	Ni	Zn	Ca	Fe	Mg	Mn	Co	K	P
	mg kg ⁻¹	dry soil							
Control	1.55 ^a	0.47 ^a	152.6 ^a	0.07 ^b	103.4 ^a	40.7 ^a	0.24 ^a	20.2 ^a	0.99 ^a
<i>S. macro.</i>	1.63 ^a	0.43 ^a	124.9 ^b	0.06 ^b	80.0 ^c	33.2 ^{bc}	0.17 ^b	15.1 ^b	0.76 ^a
<i>M. liq.</i>	1.39 ^a	0.50 ^a	151.1 ^a	0.24 ^a	90.7 ^b	28.1 ^c	0.20 ^b	19.5 ^a	0.47 ^a
<i>M. arab.</i>	1.65 ^a	0.51 ^a	148.8 ^a	0.12 ^a	113.7 ^a	47.5 ^a	0.29 ^a	17.7 ^a	0.32 ^a

S. macro., *Sphingomonas macrogoltabidus*; *M. liq.*, *Microbacterium liquefaciens*; *M. arab.*, *Microbacterium arabinogalactanolyticum*. *Means followed by different letters within the same column are significantly different at $P < 0.05$.

[036] In nonsterile soil, *Microbacterium arabinogalactanolyticum* increased Ni extractability but had no effect on any other elements examined. The concentration of extractable Ni was increased from a control of 2.2 mg kg⁻¹ to 2.6 mg kg⁻¹ when the soil was inoculated with *M. arabinogalactanolyticum*. *Sphingomonas macrogoltabidus* decreased the amount of Ni and K extracted from soil, as shown in Table 4. *Microbacterium liquefaciens* had no effect on Ni extraction in this test, but was shown to increase extraction of Fe, Mn and Co. Table 4 shows the effect of different bacterial isolates on metal solubilization in nonsterilized Ni rich soils.

Table 4

Isolate	Ni	Zn	Ca	Fe	Mg	Mn	Co	K	P
	mg kg ⁻¹	dry soil							
Control	2.2 ^b	0.48 ^a	177.4 ^a	0.012 ^b	114.2 ^{ab}	3.6 ^{bc}	0.000 ^b	21.4 ^a	0.85 ^a
<i>S. macro.</i>	1.7 ^c	0.50 ^a	17.14 ^a	0.001 ^b	103.3 ^b	0.08 ^c	0.002 ^b	16.7 ^b	0.46 ^a
<i>M. liq.</i>	1.9 ^{bc}	0.53 ^a	162.5 ^a	0.312 ^a	10.35 ^b	16.3 ^a	0.106 ^a	23.4 ^a	0.87 ^a
<i>M. arab.</i>	2.6 ^a	0.61 ^a	163.8 ^a	0.000 ^b	124.4 ^a	5.7 ^{bc}	0.000 ^b	20.7 ^a	0.421 ^a

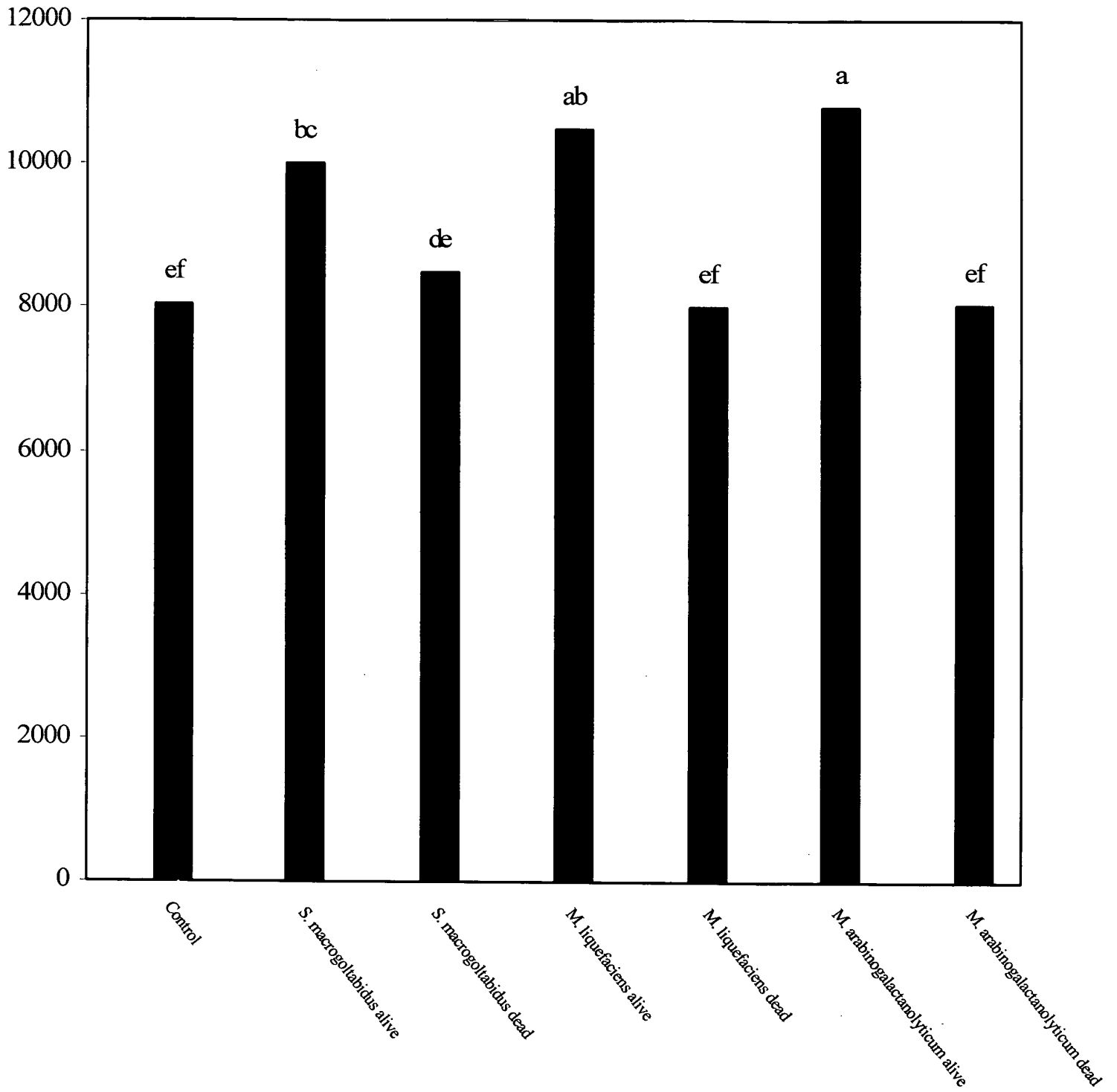
S. macro., *Sphingomonas macrogoltabidus*; *M. liq.*, *Microbacterium liquefaciens*; *M. arab.*, *Microbacterium arabinogalactanolyticum*. *Means followed by different letters within the same column are significantly different at $P < 0.05$.

[037] It is speculated that acid, siderophore production and/or phosphate solubilization by the enhancing-bacteria facilitated metal solubility in the nonsterile, Ni-rich soils; however, the invention is not limited to any particular theory. Siderophore production can also be stimulated by the presence of heavy metals (as discussed by van der Lelie *et al.*, 1999) and because most siderophores show affinity to bivalent metal ions (Neilands, 1981), they possibly affect bioavailability as well. For instance, it was reported that in *Azobacter vinelandii*, siderophore production is increased in the presence of Zn (II), as discussed by Huyer & Page, 1988. Iron chelating hydroxamic acid production in *Bacillus megaterium* is

increased by exposure to Cu, Cr, Cd, Zn, and Al (discussed by Beyers *et al.*, 1967, and by Hu & Boyer, 1996). Zinc, Cd, Ni, and Al were found to increase siderophore production in *Pseudomonas aeruginosa* (Gilis, 1993; Hassan, 1996). The same effect was found for Zn and Al in *P. fluorescens* ATCC17400 (Gilis, 1993).

[038] Bacterial inoculation on Ni uptake by *A. murale* in sterilized and nonsterilized Ni-rich soils showed that *A. Murale* grown from seeds inoculated with enhancing-bacteria (for example, the bacteria was added to the surface of the seeds) extracted more nickel than seeds that were not inoculated. To study the effect of bacterial inoculation on Ni accumulation in plants grown in nonsterile soil, *S. macrogoltabidus*, *M. liquefaciens*, or *M. arabinogalactanolyticum* were added to surface sterilized *A. murale* seeds at the time of sowing. The addition of the bacteria to the seeds significantly increased the Ni uptake of *A. murale* by 17, 24, and 32%, respectively, compared with uninoculated seeds, as shown in Chart 1. *Microbacterium arabinogalactanolyticum* increased foliar Ni from a control concentration of 8500 mg kg⁻¹ to 12 000 mg kg⁻¹. *Sphingomonas macrogoltabidus* increased Ni uptake into *Alyssum*, although to a lesser concentration as compared to the control, despite the finding that it actually decreased the concentration of Ni extracted from soil. This demonstrates that plant bioavailability of Ni was affected in a way that did not always affect extraction with a weak acid.

Chart 1



[039] There were no significant effects on uptake when dead bacteria of each isolate were tested as shown in Chart 1. Further, there were no significant effects of bacterial inoculation (dead or living) on shoot dry weight of *A. murale* (data not shown).

[040] Clearly, despite the fact that *Alyssum* hyperaccumulates Ni even without bacteria present in its rhizosphere, adding enhancing-bacteria to the soil helps to increase the extraction of Ni for hyperaccumulation from soils with a high proportion of non-labile Ni. This indicates that the bacteria somehow enhanced the availability of Ni to the plant, which in the preferred embodiment is *A. murale*. A possible explanation might be acid, siderophore production and/or phosphate solubilization although, as previously stated, the invention is not limited to a particular theory. These findings parallel Whiting *et al.* (2001), who found that the addition of a mixed inoculum of *Microbacterium saperdae*, *Pseudomonas monteilii*, and *Enterobacter cancerogenes* to surface sterilized seeds of *T. caerulescens* sown in autoclaved soil increased the Zn concentration in shoots 2-fold compared with axenic controls; the total accumulation of Zn was enhanced 4-fold.

[041] Enhanced uptake of Ni by *A. murale* is important to the overall process of phytoextraction of Ni from soils. As the technology of Ni phytomining matures and is commercially developed, even small increases in Ni uptake can have very significant impacts on profitability.

[042] Having now described a preferred embodiment of the invention, alterations and modifications that do not depart from the spirit thereof may occur to others. The invention is thus not limited to the preferred embodiments but is instead set forth in the appended claims and legal equivalents thereof. Moreover, for any method claimed herein, the steps may be performed in any manner capable of

increasing the extraction of metal from soil by a plant. Additionally, use of the indefinite article “a” means “one or more.”